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Multiplex PCR assay for molecular identification of *Brucella* strains including the vaccinal strains and its differentiation from Yersinia O: 9

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ABSTRACT

In this comparative study, five *Brucella* strains were morphologically, serologically, and biochemically identified using media different stains and antibiotics. Multiplex PCR was used to differentiate between the *Brucella* strains and *Yersinia enterocolitica O: 9*. five primer sets were designed to the most specific variable regions of the different *Brucella* species. The results of multiplex PCR were very successful and accurate in terms of characterization and typing of the *Brucella* vaccine, reference and wild *Brucella* strains from *Yersinia* which gave false positive while using tests based on anti-LPS antibodies but with PCR for DNA of *Yersinia* gave negative. The molecular typing of the *Brucella* strains by multiplex PCR had several advantages over the use of the conventional methods being very fast, precise, easier, more sensitive, and economic and could be applied on minimal sample preparation. The development of this PCR method is the first step toward the development of a novel kit for the molecular identification of *Brucella* strains from other Gram- negative bacteria.

1. INTRODUCTION

Brucellosis is caused by a facultative intracellular bacterium of the genus *Brucella*. *Brucella* is virulent mainly due to their ability to avoid the bactericidal phagocyte functions and to proliferate within macrophages, leading to the establishment of a chronic infection in the host. Brucellosis is a major zoonotic disease widely distributed in both animals and humans (Delvecchio et al., 2002), especially in developing countries, where the control programs have not succeeded. The disease is a major cause of direct economic losses, so efforts have been made to prevent the disease through the use of vaccines. Similarly, control of brucellosis is dependent upon reliable methods for the identification of *Brucella* in livestock and humans. The genus *Brucella* consist of six recognized species, designated on the basis of differences in pathogenicity and host preference: *B. melitensis* (goats and sheep), *B. abortus* (cattle and bison), *B. Sui* (swine), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (wood rats) (Corbel, 1985). The discovery of *Brucella* in a wide variety of marine mammals has led to the proposal of new species: *B. Ceti* and *B. pinnipedialis* (Foster et al., 2007). Some of species include several biovar, which are currently distinguished from each other by an analysis of approximately 25 phenotypic characteristics, including requirement for CO_2, H_2S production, sensitivity to dyes and phages, and other metabolic properties (Alton et al., 1988). However, all these tests are time-consuming, require skillful technicians and some of the essential reagents are not commercially available. In addition, handling of this microorganism represents a high risk for laboratory personnel, since most *Brucella* strain are highly pathogenic for humans. Accurate diagnostic and typing procedures are critical for the success of the eradication and control of the disease, and therefore the identification of the different species is of great epidemiological importance. The serological procedures being used in the diagnosis of animal brucellosis are mainly based on the detection of antibodies directed against the lipopolysaccharide (LPS) portion of the cell membrane. Therefore, it is difficult to differentiate between vaccinated and infected animals. In addition, tests based on anti-LPS antibodies give false positives because of cross-reactivity with other Gram-negative bacteria like *Yersinia* O: 9, *Salmonella* species, *Escherichia coli* (Corbel, 1985; KittelBerger et al., 1995 and Weynants et al., 1996). To overcome most of these difficulties, this study was designed for using a multiplex PCR assay for molecular typing of *Brucella* species. However, one of the challenges of using DNA –based

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techniques for differentiating the various *Brucella* species and strains is their high degree of genetic homology (OIE. 2008), this article describes the evaluation of a multiplex PCR.

2. MATERIAL AND METHODS

Bacterial strains:
Three lyophilized vaccinal strains [*B. abortus* biovar 1 (S19 and RB51) and *B. melitensis* biovar 1 (Rev-1)] and one reference strain [*B. melitensis* biovar 1 (16M) were supplied by CITA Institute, Zaragoza, Spain, a field isolate *B. melitensis* biovar 3 that has been isolated from an infected ewe and *Yersinia enterocolitica* O: 9 was kindly provided by the *Brucella* Department, Animal Health Research institute, Dokki, Giza. Typical *Y. enterocolitica* colonies have a" bull's eye" were chosen (UK Standard for Microbiology Investigation, 2015). The lyophilized vaccines were reconstituted in 4 ml vaccine diluent were forwarded to genomic DNA extraction, while reference strain and local field isolate were grown separately on trypticase soya agar enriched with 0.1 % yeast extract at 37 °C for 48 hours in 5% CO_2 atmosphere and *Y. enterocolitica* O: 9 was grown on Cefsulodin, Irgasan, *Novobiocin* (CIN) agar at 30 °C for 24-48 hrs. A pure culture of each strain was harvested in normal saline and pelleted by centrifugation (1700 ×g), the pellet of each strain was washed twice with 0.85% normal saline to ready for DNA extraction. The abovementioned strains were identified morphologically, biochemically and serologically. Moreover, they have been grown on media containing different stains and antibiotics (Alton et al., 1988) to identify vaccinal strains from other *Brucella* reference strain and field isolate and the results were compared with those obtained by multiplex PCR method.

Extraction of genomic DNA from Bacterial strains:
Genomic DNA extraction was performed using Wizard Genomic DNA purification kit (Promega, USA).

Multiplex PCR method:
In this study, a multiplex PCR assay was performed according to (Dubray et al., 1985). Five primer pairs (Bioneer, Germany) (Table 1), designed on the strain specific genetic differences, were used in multiplex for molecular typing of different *Brucella* species and it was used to differentiate between the *Brucella* strains and *Yersinia enterocolitica* O: 9. In this study six multiplex PCR reaction mixtures each of 25 µl volume containing 1 µl of template DNA, 200 µM of each deoxynucleoside triphosphate (Fermentas), 2.5 units of Dream Taq Green DNA polymerase (Fermentas), 5 µl of its amplification buffer, and 20 pmole of each primer, were added. The PCR amplification was carried out using Gene Amp. PCR system 9700 thermal cycler (Applied Bio systems, USA). The cycling condition were 7 minutes at 95 °C for initial denaturation, 25 cycles each of 30 seconds at 94 °C for denaturation, 30 seconds at 64 °C for primer annealing, 1 minute and 40 seconds at 72 °C for extension of the amplicons, and one cycle at 72 °C for 7 minutes for final extension. The PCR amplicons were analyzed by running 10 µl of the PCR products in 1% agarose gel stained with ethidium bromide (0.5 µg/ml). Amplification pattern of each *Brucella* species was determined according to molecular size of the amplified products (Figure 1). Whole genome sequences of *B. melitensis* (Corbel, 1988) and B. *abortus* (Garcia-Yoldi, 2006) were used.

3. RESULTS

In the present study, five *Brucella* strains (one local isolate, three vaccinal strains and one reference virulent strain) have been identified using traditional and recent methods. The local field isolate was recovered from ewe clinically diagnosed as brucellosis. The vaccinal strains were *Brucella abortus* S19, RB51 and *Brucella melitensis* Rev-1 which represent live attenuated vaccines against brucellosis. The one reference virulent strain was *B. melitensis* 16 M biovar1. The five strains were traditionally identified using the most common methods, whereas the ~~cultivation~~ cultural characters were consistent with the typical characters of *Brucella* species. All strains grew within 3 days as raised, convex, circular colonies with smooth surface. The vaccinal strain RB51 showed rough colonies which take red stain when stained with crystal violet and can resist and grow in media containing rifampicin (250 µg/ml media).

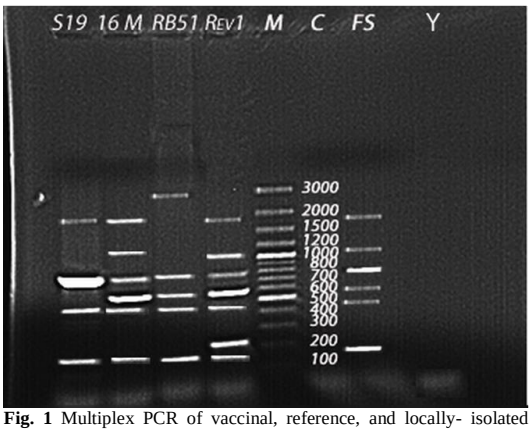

Fig. 1 Multiplex PCR of vaccinal, reference, and locally- isolated Brucella strains. M (marker) =100 base pair (bp) DNA ladder (Ferments). Lanes 1, 2,3,4,5 are Bruce- Ladder multiplex PCR amplicon of different *Brucella* species. Multiplex PCR of *B. melitensis 16M* reference strain in (lane 1), *B. melitensis Rev1* vaccinal strain (lane 2) and the *B. melitensis* (biovar 3) field strain isolated from ewe (lane 5) produced six amplification products of 1682, 1071,794, 587,450 and 152bp in size with an additional 218 bp sized fragment in *Rev 1* strain only. *B. abortus RB51* vaccinal strain (lane 3) showed amplicons of 2524 bp fragment in *RB51* strain only. *B. abortus S19* vaccinal strain (lane 4) did not produce the 587 bp fragment common to all species. PCR for DNA of Yer*sinia enterocolitica O: 9* give negative (lane 6).

The vaccinal strain S19 revealed smooth colonies which have not been stained with crystal violet and could not grow on media containing thionin blue (20 µg/ml), erythritol (1 mg/ml) and penicillin (5 IU/ml) in contrast to other strains. On the other hand, the vaccinal strain Rev-1 produced smooth colonies which have not been stained with crystal violet and could not grow on media containing thionin (20 µg/ml), basic fuchsine (20 µg/ml and penicillin (5 IU/ml) but grew on media containing streptomycin (2.5 µg/ml), in contrast to other strains. The biochemical method confirmed that S19 and RB51, each behaved as *B. abortus* while Rev-1, 16M strains were *B. melitensis*-like in their biochemical tests.

Table 1 Primer sets for multiplex PCR.

Primer	Sequence (5· _3)	Amplicon Size (bp)	DNA targets	Source of genetic difference
BME10998F	ATC-CTA-TTG-CCC-CGA-TAA-GG	1682	Glycosyl transferase, gene wboA	S711 insertion in *B. abortus* RB51.
BME10997R	GCT-TCG-CAT-TTT-CAC-TGT-AGC			
BMEII0843F	TTT-ACA-CAG-GCA-ATC-CAG-CA	1071	Outer membrane	deletion of 25,061
bp in			Protein, gene omp 31	BMEII826-
BMEII0850				
BMEII0844R	GCG-TCC-AGT-TGT-TGT-TGA-TG			in *B. abortus*
BMEII0428F	GCC-GCT-ATT-ATG-TGG-ACT-GG	587	Erythritol catabolism, gene ery C (D-erythrulose	deletion of 702 bp in BMEII0427 -
BMEII0428				
BMEII0428R	AAT-GAC-TTC-ACG-GTC-GTT-CG		-1-phosphate dehydrogenase)	in *B.abortus S 19*
BR0953F	GGA-ACA-CTA-CGC-CAC-CTT-GT	272	ABC transporter binding	deletion of 2653 bp
in			Protein	BR0 951 BR 0955 in *B.*
abortus				
BR0953R	GAT-GGA-GCA-AAC-GCT-GAA-G			and *B. melitensis*
BMEI0752F	CAG-GCA-AAC-CCT-CAG-AAG-C	218	Ribosomal protein S12, gene rpsL	point mutation in *B.melitensis Rev.1*
BMEI0752R	GAT-GTG-GTA-AGG-CAC-ACC-AA			

Table 2 Species and biovar biochemical and morphological differentiation of the *Brucella* strains.

	Brucella Spp.				
	Brucella abortus		*Brucella melitensis*		
	S19	RB51	Rev 1	16 M	Field isolate
Morphology	Gram	negative	Small	Coccobacilli	
Lactose fermentation on MacConkey agar	-	-	-	-	-
Hemolysis on blood agar	-	-	-	-	-
Catalase test	+	+	+	+	+
Nitrate reduction test	+	+	+	+	+
Urease test	+	+	+	+	+
Oxidase test	+	+	+	+	+
Colonial morphology	Smooth	Rough	Smooth	Smooth	Smooth
CO_2 requirement	-	+	-	-	-
H2S production	+	+	-	-	-
Growth on dyes					
1-Thionin 1:50,000 (20 µg/ml)	+	+	-	+	+
2-Basic fuchsine 1:50,000 (20 µg/ml)	+	+	-	+	+
3-Thionin blue (2 µg/ml)	-	+	+	+	+
Growth in the presence of erythritol (1 mg/ml)	-	+	+	+	+
Growth in the presence of antibiotic					
1- Streptomycin (2.5 µg/ml)	-	-	+	-	-
2- Penicillin (5 IU/ml)	-	+	+	+	+
3- Rifampicin (250 µg/ml)	-	+	-	-	-

Table 3 Species and biovar serological differentiation of the collected *Brucella* strains.

Brucella species	Monospecific antisera		
	A	M	R
B. abortus S-19 (biotype 1)	+	-	–
B. abortus S-RB51(biotype 1)	+	-	+
B. melitensis Rev-1(biotype 1)	-	+	-
B. melitensis 16 M(biotype 1)	-	+	-
B. melitensis local field isolate (biotype 3)	+	+	-

However, Egyptian field isolate was identified as *B. melitensis* according to biochemical identification (Table 2 and 3). Three monospecific antisera (A, M and R) were used in identification. Where strain 19 cross-reacted with the strain RB51 monospecific antiserum A as it is *B. abortus*, the vaccinal strain RB51 reacted with the monospecific antiserum R. The Rev-1 and 16M strains agglutinated with the monospecific antiserum M as they are *B. melitensis* biovar1. The field isolate agglutinated with both M and R monospecific antisera which indicated that this strain is *B. melitensis* biovar3. Molecular characterization trials have been carried out to differentiate the different *Brucella* species and biovar. DNA dependent methods have been recently nominated as tools for identification and differentiation of *Brucella* isolates. While lots of methods were used for genomic characterization of *Brucella*, the restriction fragment length polymorphism (RFLP) and PCR were the most commons. *Yersinia enterocolitica* O: 9 was cultured on Cefsulodin, Irgasan, and Novobiocin (CIN) agar at 30 °C for 24-48 hrs prior to DNA extraction. Typical *Y. enterocolitica* colonies having a "bull's eye" were chosen. Detection of the unique genomic differences of *Brucella* species were based on published whole genome sequences of *B. melitensis* and B. *abortus* used in this study (Table 1). *Brucella* species were differentiated based on the size of the produced amplicons. In Multiplex PCR assay using DNA from *B. melitensis* strains, six fragments were amplified: of 1,682, 1,071,794,587,450 and 152 bp in size with an additional 218 bp sized fragment in Rev-1 strain only produced by the BMEI0752 primer pair (Table 1). PCR using DNA from *B. abortus* strains amplified five fragments, of 1,682, 794,587,450 and 152 bp in size. On the other hand, *B. abortus* RB51 vaccinal strain did not produce the 1682 bp fragment due to disruption of the wboA gene by an IS711 element in *B. abortus* RB51. In addition the aforementioned results led to the conclusion that the multiplex PCR has successfully identified as well as differentiated each of the tested *Brucella* species and the vaccine strains in the same test and also to differentiate between *Brucella* strains and *Y. enterocolitica* O: 9 which give negative. In addition, a multiplex PCR assay confers several advantages over the current identification methods the major advantage is the speed with which the assay can be performed together with minimal sample preparation, whereas only 104 bacteria can be added directly to the reaction mixture.

4. DISCUSSION

Identification of different members of Brucella has been done with the traditional methods such as cultural, biochemical characterization and serological identification (Alton et al., 1988). Recent methods were also introduced to the field of laboratory identification of Brucella (Godfroid et al., 2002). In the present study, five Brucella strains (one local isolate, three vaccinal strains and one reference virulent strain) have been identified using traditional and recent methods; the local field isolate was recovered from ewe clinically diagnosed as brucellosis. All strains grow within 3 days as raised, convex, circular colonies with smooth surface. Gram and Modified Ziehl-Neelsen stained films showed Gram-negative or pink, weak acid fast short rods with round ends and slightly convex sides (Jensen et al., 1995). The vaccinal strain RB51 showed rough colonies which take red stain when stained with crystal violet and can resist and grow in media containing rifampicin (250 µg/ ml media). These results were consistent with that recorded by Alton et al. (1988) and Jensen et al. (1995).

In the present study, genomic DNA was extracted and a multiplex PCR assay was used to identify as well as to differentiate Brucella vaccinal strains from other Brucella species and biovar, besides a field isolate and to differentiate between Brucella strains and Enterocolitica O: 9. Previously designed five multiplex primer sets (Dubray, 1985). Typical Y. enterocolitica colonies having a" bull's eye" were chosen. Detection of the unique genomic differences of Brucella species was based on published whole genome sequences of B. melitensis (Corbel, 1988) and B. abortus (Garcia-Yoldi, 2006) were used in this study. PCR of RB51 was characterized by a specific additional band of 2524 bp size, a similar result was reported by KittelBerger et al. (1995) and Halling et al. (2005). The BMEI0752 primer pair detected a unique point mutation in the rpsL gene, coding for the Ribosomal protein S12, of the vaccinal strain B. melitensis Rev-1 and responsible for Streptomycin resistance of Rev-1 (Cloeckaret et al., 2002). PCR with B. abortus S19 did not produce the 587 bp fragment, common to all Brucella species due to deletion of 702 bp in the Erythroid catabolism gene eryC (Lopez-Goni et al., 2008) leading to miss priming of the BMEI0428 primers pair. On the other hand, B. abortus RB51 vaccinal strain did not produce the 1682 bp fragment due to disruption of the wboA gene by an IS711 element in B. abortus RB51.

5. CONCLUSION

Multiplex PCR assay is recommended for testing the seed cultures commonly used in the production of live *Brucella* vaccines (*Rev-1, S19* and *RB51* vaccines) and in evaluating them in quality control laboratories.

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